Effects of Ca^{2+} concentration and alginate cross-linking and chondrocyte differentiation.

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INTRODUCTION

Cartilage is the connection tissue cushion between the bones, providing support and relieving stress on a joint. Relatively avascular and cell-free, cartilage is slow to grow and repair. Thus, it is of interest to engineer an implant to regenerate this tissue.

Tissue engineering is comprised of three main components: the scaffold, the cells to be seeded on the scaffold, and soluble factors that will affect cell behavior. There are many characteristics – such as porosity, elasticity, degradability, etc. – of the scaffold that can be altered and tuned for specific goals. In addition, a number of cells could be grown within the scaffold; for this specific experiment, the options were chondrocytes (which produce the proteins present in the extracellular matrix of cartilage) or mesenchymal stem stells (which are the progenitors of chondrocytes). Lastly, any number of external chemical factors could impact cell growth and differentiation within the scaffold.

When grown in monolayer, chondrocytes dedifferentiate back into fibroblasts; but mesenchymal stem cells also have the potential to differentiate into collagen-producing chondrocytes. When cultured in alginate and collagen type I and II hydrogels, mesenchymal stem cells underwent chondrogenesis (Bosnakovski 2006). Chondrocytes grown on a less stiff scaffold, created by fewer crosslinks within the scaffold, also showed a circular shape, more typical of the chondrocyte phenotype (Genes 2004). A greater cell seeding density was found to stabilize chondrocyte phenotype (Domm 2004). From these experiments, it could be reasoned that culturing chondrocytes on three-dimensional alginate scaffolds of varying porosities would influence differentiation and collagen production.

For our experiment, we chose to seed chondrocyte cells at a density of $5x10^6$ cells/mL onto FMC Biopolymer Pronatal LF 10/60 alginate scaffolds in solutions with a calcium concentration of 50 mM and 1000 mM. We expect the cells to be more differentiated in the scaffolds produced in higher calcium concentrations, which produce scaffolds with more cross-links, since three-dimensional scaffolds promote chondrogenesis.

RESULTS

Fluorescence Microscopy

Scaffolds formed under 50 mM [Ca^{2+}] and 1000 mM [Ca^{2+}] were stained with fluorescent dyes and imaged under fluorescent excitation in order to assess cell viability (Figure 1). Cells fragments were seen in the 1000 mM [Ca^{2+}] scaffold under normal light, but no cells showed up under fluorescent excitation. Clusters of cells were found in the 50 mM [Ca^{2+}] scaffold under normal light, which then appeared green under fluorescent excitation. These results indicate that the cells were in the 50 mM [Ca^{2+}] scaffold were still alive, while cells in the 1000 mM [Ca^{2+}] scaffold were either all dead or unable to take in the fluorescent dye.

Cell Density

Cells were counted after a week of culture in scaffolds. The 50 mM [Ca^{2+}] scaffold had a cell density of $1.6x10^5$ cells/mL while the 1000 mM [Ca^{2+}] scaffold had a cell density of $7.8x10^5$ cells/mL.

RT-PCR

RNA was extracted from the scaffolds, converted to cDNA, and then amplified using reverse transcriptase polymerase chain reaction. Purity of the RNA extract was assessed by measure absorbance of the samples diluted 1:100 at 260 and 280 nanometers; the ratio of A_{260} to A_{280} was essentially 1 (0.019/0.018 for the 50 mM [Ca^{2+}] sample, 0.013/0.015 for the 1000 mM [Ca^{2+}] sample), which indicates that the sample was relatively impure (ratio above 2 is ideal). Therefore, 300 ng of RNA (rather than the normal 100 ng) from each sample underwent the RT-PCR reaction.

Gel Electrophoresis

The amplified cDNA was run through an agarose gel to isolate DNA fragments corresponding to the collagen types I and II transcripts. Lane 1 contained a 100 bp ladder; lane 2 contained the 50 mM [Ca^{2+}] sample targeting collagen I; lane 3 contained the 1000 mM [Ca^{2+}] sample targeting collagen I; lane 4 contained the 50 mM [Ca^{2+}] sample targeting collagen II; lane 5 contained the 1000 mM [Ca^{2+}] sample targeting collgen II.

The electrophoresis gel (Figure 2) revealed bands at the expected size of collagen I, collagen II, and GAPDH, which indicate the presence of the corresponding mRNA transcripts in both samples. GAPDH, used as an internal control, should have similar expression in all four samples; however, GAPDH expression was much higher in the samples targeting collagen II. Due to this finding, it is difficult to extrapolate any conclusions from this gel; however, we continued with our analysis. Using ImageJ, a program provided by the National Institutes of Health, we measured the intensities of each band in the gel. However, the image was taken at a very low exposure time, probably because we loaded so much RNA into the RT-PCR reaction that resulted in a high amount of DNA loaded into the gel electrophoresis. The intensity values from the bands corresponding to collagen I and II expression levels for both the 50 mM and 1000 mM [Ca^{2+}] samples were background corrected and normalized to their appropriate GAPDH band intensities. Because the background-corrected and normalized intensity of the 50 mM [Ca^{2+}] sample targeting collagen I was 0, it is meaningless to compare collagen type I and II expression between the 50 mM and 1000 mM [Ca^{2+}] samples.

ELISA Assay

An ELISA assay was performed to compare amounts of collagen types I and II protein present in the cells. The primary antibody targeted either collagen type I or II, while the secondary antibody targeted the primary antibody used. We loaded PBS as a control, and duplicate protein samples at known concentrations to create a standard. A reaction that turns the sample yellow relative to the amount of target protein allowed us to calculate the initial amount of collagen present in the sample by measuring OD at 420 nm. Values were background-corrected, and the standard values were graphed against their known protein concentrations to create a trendline from which we could calculate sample concentrations of collagen (Figure 3); the three highest values were removed from the data set in order to preserve the linear trendline.

For the samples targeting collagen I, we got background-corrected OD_{420} values of 0.045 for the 50 mM [Ca^{2+}] sample and 0.002 for the 1000 mM [Ca^{2+}] sample. Using the trendline, we calculated collagen I concentrations of 0.05 μg/mL and 0 μg/mL for the 50 mM and 1000 mM [Ca^{2+}] samples, respectively. For the samples targeting collagen II, we got background-corrected OD_{240} values 0.002 for the 50 mM [Ca^{2+}] sample and 0.040 for the 1000 mM [Ca^{2+}] sample. This results in collagen II concentrations of 0 μg/mL and 0.03 μg/mL for the 50 mM and 1000 mM [Ca^{2+}] samples, respectively. The higher collagen II concentration in the 1000 mM sample

indicates that the denser scaffold helped maintain chondrocyte phenotype better than the more porous scaffold created at lower calcium concentrations.

DISCUSSION

Because so many parts of the experiment had issues, it is difficult to draw conclusions from the results collected. A reiteration of this experiment would allow verification our data, or perhaps new conclusions to be drawn.

When assessing the viability of cells within the scaffold, we found no stained cells when viewing the 1000 mM [Ca^{2+}] scaffold under fluorescent excitation. This led to the conclusion that the 1000 mM [Ca^{2+}] scaffold was just too stiff or dense to allow cells to survive within the construct; however, the electorphoresis gel and ELISA assay showed the presence of mRNA transcripts and collagen proteins in the extracts from the 1000 mM [Ca^{2+}] scaffolds. It is conceivable that the three alginate beads we chose to image happened to not contain any cells; however, this hypothesis is rather unlikely.

The data from the gel electrophoresis after RT-PCR of extracted RNA also seems rather problematic. Expression of GAPDH, our internal control, is expected to remain constant in all samples since the same amount of RNA was loaded into the RT-PCR reaction for both samples. However, there was a significantly higher intensity for bands corresponding to samples that targeted collagen II, including the GAPDH bands. This error points to a human error during the RT-PCR reaction, which was the only step at which samples for collagen I and II were ever separated. Because of this error, it would not be useful to draw any conclusions from our gel.

In addition, our results from the ELISA assay do not appear to make sense. Concentrations of collagen I are expected to be the same in both the 50 mM and 1000 mM [Ca^{2+}] samples since both chondrocyctes and dedifferentiated chondrocytes (i.e. fibroblasts) produce collagen I; however, this was not the case. The difference in collagen I concentrations renders any other conclusions or calculations performed from the ELISA assay data rather suspicious. In addition, the OD_{240} values for the experimental samples were below those of the standard concentrations and close to the background noise, which didn't allow us significant values with which we could calculate collagen concentrations

Despite these issues, we concluded that varying calcium concentration to affect scaffold porosity did indeed have an effect on chondrocyte phenotype and differentiation. It is difficult to say whether the higher calcium concentration definitively aided in maintaining the chondrocyte phenotype; further experimentation or a repeat of this experiment would shed more light on this topic. In the future, more experimental samples to allow a wider range of calcium concentrations would provide more information about scaffold porosity and its effect on chondrocyte differentiation.

REFERENCES

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Domm C, Schünke M, Steinhagen J, Freitag S, Kurz B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. Tissue Engineering 2004; 1796-1805.

Genes NG, Rowley JA, Mooney DJ, Bonassar LJ. Effect of substrate mechanics on chondrocyte adhesion to modifiifed alginate surfaces. Archives of Biochemistry and Biophysics 2004; 161-167.

FIGURES

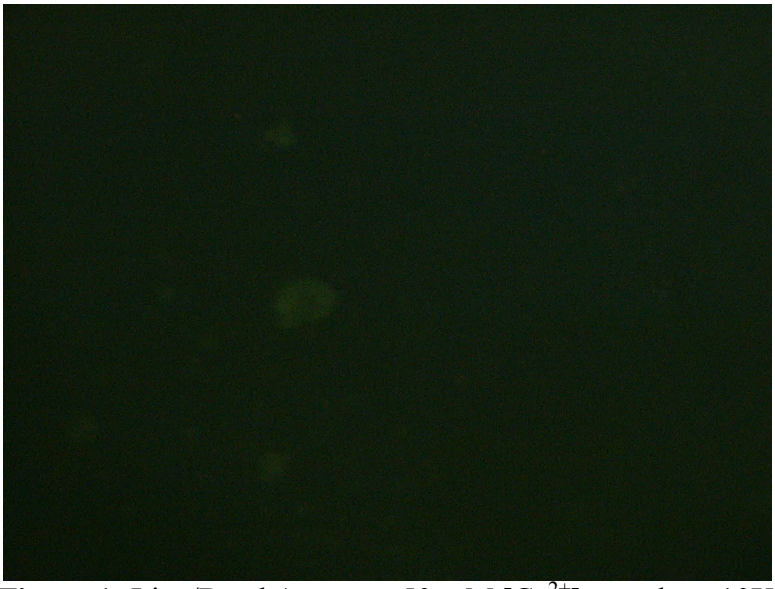


Figure 1: Live/Dead Assay on 50 mM [Ca^{2+}] sample at 10X magnification.

Fluorescence microscopy of one alginate bead from 50 mM [Ca^{2+}] sample after staining with fluorescent dyes. Live cells are green while dead cells are red.

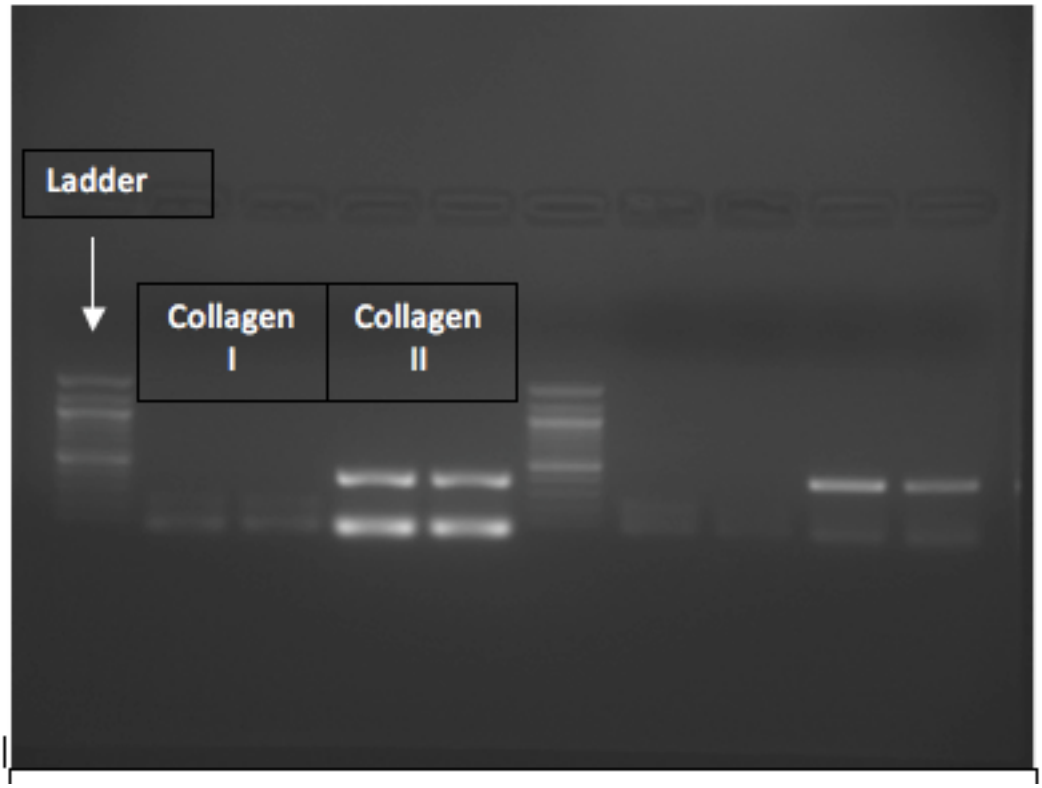
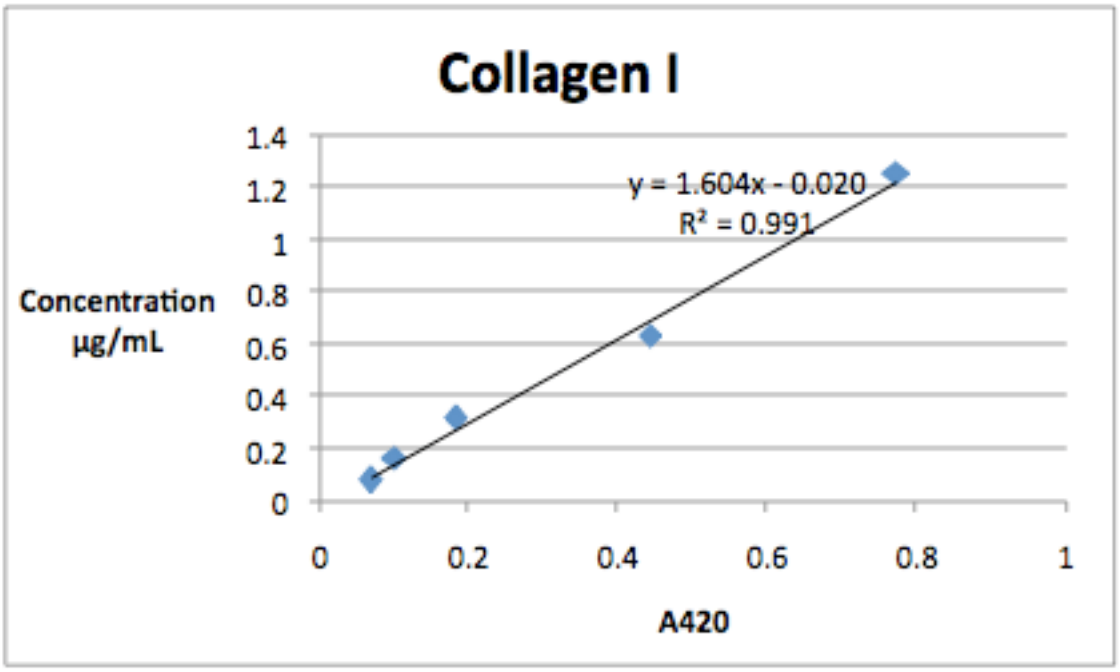


Figure 2: Electrophoresis gel of RT-PCR products.

Lanes: (1) 100 bp ladder, (2) 50 mM [Ca^{2+}] sample targeting collagen I, (3) 1000 mM [Ca^{2+}] sample targeting collagen I, (4) 50 mM [Ca^{2+}] sample targeting collagen II, (5) 1000 mM [Ca^{2+}] sample targeting collagen II. The first band corresponds to collagen I in lanes 2-3 and collagen II in lanes 4-5. The second band in lanes 2-5 corresponds to GAPDH.



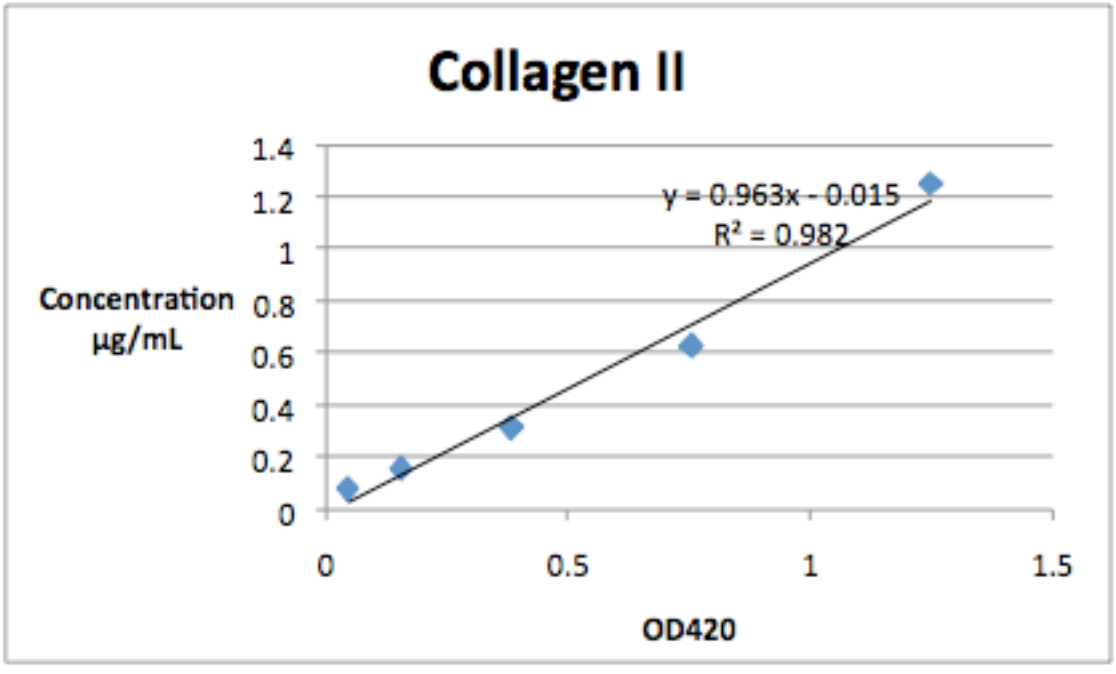


Figure 3: Collagen I and II graphed standards and computed trendlines.